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14. ABSTRACT

Rho GTPases are molecular switches that fluctuate between on and off states. When active, these proteins function to remodel the actin cytoskeleton by interacting with a number of downstream effector molecules. Recent studies have linked the activation of Rho GTPases with the acquisition of a metastatic phenotype in many types of cancers especially breast cancer. This proposal incorporates a rational approach to target these signaling proteins using small molecule inhibitors that would interfere with their ability to become activated by Rho family guanine nucleotide exchange factors (RhoGEFs). We have developed a high throughput screening strategy identify novel inhibitors of Rho activation are currently following up on several compounds which appear to selectively inhibit Rho activation. These compounds may form the basis of future drug development strategies for the treatment of metastatic breast cancer.

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Introduction

Rho GTPases belong to the Ras superfamily of small (~21 kDa) monomeric guanine nucleotide-binding proteins. There are approximately 20 Rho subfamily members, the most characterized of which are RhoA, Rac1, and Cdc42. Like other guanine nucleotide binding proteins, Rho family members are molecular switches that fluctuate between active GTP-bound and inactive GDP-bound states. Rho GTPases are signaling molecules that can propagate signal transduction events initiated by extracellular stimuli and have been shown to promote an invasive and metastatic phenotype in a variety of cancer types via cytoskeletal rearrangement, as shown in Fig. (**1**). For example, activation of receptor tyrosine kinases, G-protein coupled receptors, or integrin receptors results in the activation of a Rho Guanine nucleotide Exchange Factor (RhoGEF), which facilitates the GTP-loading of specific Rho GTPases via the catalytic Dbl-homology (DH) domain [for reviews, see 1-5]. Once GTP-bound, Rho GTPases adopt an active conformation and are free to interact with downstream effector molecules, resulting in their activation. Rho GTPases are mediators of actin cytoskeletal remodeling and have been linked with the acquisition of a metastatic and invasive phenotype in several cancer types [7-19]. Recently, numerous reports have suggested that Rho GTPase activation is an integral step during the invasion and metastasis process of a wide variety of cancers including inflammatory breast cancer (IBC) [7-19]. Consequently, a recent issue of the journal *Breast Cancer Research and Treatment* was devoted exclusively to reports describing the link between Rho family GTPases and breast cancer progression and dissemination [7-13]. Additionally, a recent report has shown the Rho isoform, RhoC, is essential for metastasis in a mouse breast tumor model [22]. The authors show that RhoC-deficient mice develop normally and have proper migratory function of neutrophils, thymocytes, T-cells, and B-cells. However, when subjected to a mammary breast cancer tumor formation model, which typically gives rise to palpable tumors at ~50 days and disseminated cancer in the lungs at ~70 days, the RhoC-deficient mice have a significant reduction in the amount of metastasis in the lung. These studies, as well as numerous others, suggest that interfering directly with Rho GTPase function is an ideal strategy for controlling the dissemination of primary tumors to distant metastatic sites. However, due in a large part to the lack of appropriate screening assays, there is currently a lack of useful small molecule inhibitors of Rho GTPase function, which could be developed into therapeutics for the treatment of metastatic breast

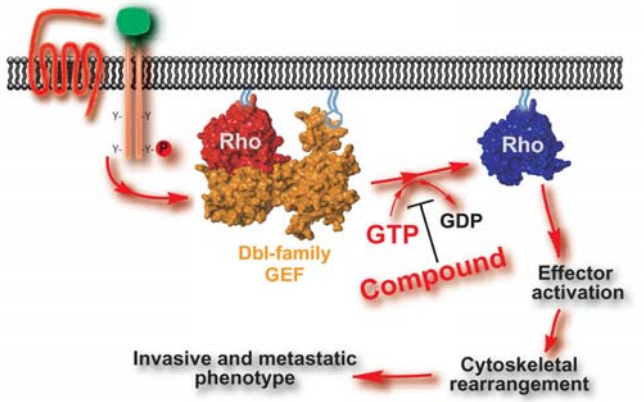

Fig. (1) Rho GTPases (Rho, Rac, Cdc42) are emerging candidates for targeted therapeutics in metastatic breast cancer; however, there are currently no compounds, which directly target the rate-limiting step of Rho activation. Activation of upstream signals, such as receptors, results in activation of Dbl-family guanine nucleotide exchange factors (GEFs) for Rho GTPases at the plasma membrane. GEFs catalyze the rate-limiting step of Rho activation by converting the inactive GDP-bound Rho and Rac into active GTP-bound Rho, which is then able to activate downstream effector molecules, resulting in cytoskeletal rearrangement, which contributes to an invasive and metastatic phenotype in many breast cancers. Our objective is to identify novel compounds, which disrupt the activation of Rho GTPases, thereby reducing the metastatic potential of cancer cells.

cancer. For these reasons, our objective is to develop more efficient methods for high throughput screening and identification of novel inhibitors of Rho GTPase activation.

Results

***In vitro* guanine nucleotide exchange assay:** In order to conduct many of the proposed experiments outlined in the statement of work, we needed to first perfect a method for evaluating potential small molecule inhibitors of Rho GTPase activation. We have focused on a fluorescence-based guanine nucleotide exchange assay that used purified components and commercially available fluorescent probes. The guanine nucleotide exchange assay makes use of the spectroscopic differences between free and protein-bound fluorophore-conjugated guanine nucleotides. Traditional methods for studying GTPase activation have relied on radiolabeled filter-binding assays, which are labor intensive, and lack precise sampling rates. However, the fluorescence-based exchange assay is more conducive for high content experiments that require precise determination of reaction rates. This is because real-time analysis, using a spectrophotometer, allows precise sampling rates required for determining the rate at which the exchange reaction occurs. While other groups routinely use this assay to characterize GTPases and GEFs, we have implemented this assay for the purposes of discovering novel inhibitors of Rho GTPase activation. This has entailed slight modifications to the original assay design, the details of which can be found in the methods section. Our assay validation studies have demonstrated that the assay is versatile enough to identify compounds with several different modes of action. By using a functional assay, as opposed to a binding assay, we are not limited by the mode of action any compound examined. Functional assays, such as the guanine nucleotide exchange assay, are therefore much more amendable to high throughput screening strategies. Additionally, we have purified a large number of Ras superfamily GTPases as well as their associated GEFs to near homogeneity for use in the guanine nucleotide exchange assay. This has entailed a high content approach to the cloning, expression, and purification of affinity-tagged protein constructs as described in the methods section. We are currently using these purified protein reagents to determine the selectivity of any identified small molecule inhibitors of Rho GTPase activation and have successfully used many in the guanine nucleotide exchange assay.

High throughput screening-NCI Diversity Set: We have also incorporated additional strategies for the identification of small molecule inhibitors of Rho GTPase activation, namely high throughput screening of 96-well plated small molecule libraries as described in the original proposal. We have obtained and subsequently screened the National Cancer Institute (NCI) Diversity Set of ~2,000 compounds. This library is freely available to investigators and is designed to represent a wide variety of diverse compounds for screening purposes. We have completed screening for chemical modulators of Tiam1-mediated activation of Rac1 using a 96-well format of the guanine nucleotide exchange assay. After the primary screening results, 32 compounds showed significant activity against Tiam1-mediated activation of Rac1. However, upon secondary analysis using non-related GEF-GTPase pairs, including Dbs-RhoA and Sos1-HRas, only one compound selectively modulated Tiam1 activity. Interestingly, this compound increases the kinetic rate of guanine nucleotide exchange and is therefore considered an activator, as opposed to an inhibitor. While our original purpose was to identify inhibitors of Rac1 signaling in order to intervene with metastatic signal transduction events, the discovery of a Tiam1 activator may prove useful as a biomolecular tool for probing Rac1-mediated signaling. NSC#13778 (phenylstibinoacrylic acid) accelerates Tiam1 activation of Rac1 in a dose-dependent manner with an EC_{50} of ~5 μM, while unaffecting Dbs-RhoA, Dbs-Cdc42, and Sos1-HRas guanine nucleotide exchange. This activity has additionally been confirmed using non-fluorescent assays such as the traditional radiolabeled nucleotide filter-binding assay. We also have completed preliminary structure-activity relationship studies with over 15 structural analogs of NSC#13778 in order to better understand which functional groups are most important for the mechanism of action.

High throughput screening-LDDN collaboration: While the NCI Diversity is a useful library for initial drug screening purposes; it is of limited size and was mainly used as a proof of principle, in order to establish that our fluorescent nucleotide exchange assay can be used to identify novel small molecules that modulate Rho GTPase activation. We have therefore formed a collaborative agreement with an academic drug-screening lab, which gives our group access to over 60,000 small molecules in microtiter format. In light of recent reports implicating Rho in breast cancer metastasis [22], we have also decided to focus more intently on identifying Rho-specific inhibitors. For this reason, our current drug screening efforts have utilized Rho-specific GEFs, such as Dbs, instead of Tiam1 as originally proposed. Additionally, in order to effective screen such a large library, we needed to slightly modify the primary assay for ultra high throughput drug screening purposes, the details of which we hope to publish in the near future. Together with Dr. Ross Stein at the Laboratory for Drug Discovery in Neurodegeneration (LDDN), Harvard University, we converted the 96-well formatted fluorescent-based guanine nucleotide exchange assay, which was originally used to screen the NCI Diversity Set, into a simplified 2-point assay for 384-well plates in a semi-automated fashion. This has entailed slowing down the reaction kinetics in order to focus on the near linear portion of the exchange reaction and boost the overall signal. The linearized exchange reaction was then further simplified to a 2-point assay, vastly reducing the amount of manipulations, sampling rate, and post-screening analysis required to interpret results. An initial test set consisting of ten 384-well plates was then used to determine the statistical parameters typically employed to validate high throughput screening assays. This test set illustrates that our method yields reproducible and statistically sound results, as the Z-factor is within the allowed range for an exceptional screening assay which is typically 0.6 - 1.0 [23]. We have completed screening ~30,000 compounds through this collaboration, most compounds are not active against Rho activation by Dbs, however 0.23% show significant activity of greater than 75% inhibition.

Training: As a predoctoral fellow in the Breast Cancer Research Program, I have received exceptional mentoring and formal training in a number of subject areas that will support my future as a cancer researcher. Foremost, my advisor John Sondek, Ph.D. has been instrumental in developing my skills in scientific inquiry by persistently challenging and encouraging my development as scientist. Aside from typical sources of instruction, which include formal group meetings and weekly seminars, my training has also included instruction in bioinformatics, computational techniques, three dimensional structure manipulation, virtual small molecule docking, protein purification techniques, high-throughput assay development, and X-ray crystallographic techniques for structure determination. This training regimen has consisted of formal training either individually from experts in the field or in a group setting in the form of course work and workshops. In addition to my advisor, thesis committee, and members of the department, I have also been closely mentored by several other outstanding researchers including the director of the UNC structural bioinformatics core facility (Brenda Temple, Ph.D.), members of the LDDN at Harvard University (Ross Stein, Ph.D. and Li-An Yeh, Ph.D.), the director of the X-ray core facility at UNC (Laurie Betts, Ph.D.), and the director of the protein core facility at UNC (Jason Snyder, Ph.D.). This training and mentorship has been invaluable to my development as a cancer researcher and has also significantly facilitated the implementation of these studies.

Methods

Protein purification: The coding sequences for all clones were introduced into a bacterial expression system as His-tagged protein constructs using standard PCR and molecular biology techniques. These clones consist of full-length sequences for the human GTPases RhoA, RhoB, RhoC, Rac1, Cdc42, and H-Ras as well as the catalytic fragments of the exchange factors Tiam1, Dbs, LARG, and Sos1. Proteins were expressed in E. coli and purified using standard Ni-NTA affinity purification methods followed by size-exclusion chromatography using an FPLC. All purified proteins were then subjected

to SDS-PAGE analysis to determine correct molecular weight and protein purity. Only proteins that were >95% pure were used for biochemical and biophysical studies.

High throughput screening of the NCI Diversity Set and analysis of NSC#13778: The publicly available Diversity Set was obtained from the NCI in a 96-well format and reconstituted in 100% DMSO at a final concentration of 10 mM. This library was used to screen for small molecule modulators (either activators or inhibitors) of Tiam1-mediated activation of Rac1 using reaction conditions described above. The screen was carried out in a microtiter, 96-well format using a Biomek FX liquid handling robot to set up the reactions and a Molecular Devices microtiter fluorimeter to monitor the reaction kinetics. All compounds tested were compared to DMSO only controls and the % inhibition was calculated by fitting each reaction to an exponential growth function using GraphPad Prizm data analysis software as described above. Secondary screening of potential hits consisted of screening compounds against an unrelated GEF-GTPase pair, such as Dbs-RhoA and Sos1-HRas in order to determine selectivity. A dose-response curve was then generated for NSC#13778 and was used to determine the EC_{50} value of ~5 μM.

High throughput screening collaborative effort with the LDDN and statistical validation of the 2-point assay: The 96-well formatted guanine nucleotide exchange assay used to screen the NCI Diversity Set was modified to suit the demands of a 384-well formatted screen of ~30,000 compounds by adjusting reaction conditions and using more sensitive instrumentation. The final reaction condition for screening compound at 10 μM consisted of 20 mM Tris pH 7.0, 150 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 50 μg/mL BSA, 200 nM Dbs (catalytic DH/PH fragment), 1.5 μM RhoA, and 640 nM Bodipy-GTP (Molecular Probes). A Biomek FX liquid handling robot was used to add reagents to a top reading, black 384-well plate (Fluotrac 200). Fluorescence intensity was measured using an Analyst HT microplate fluorimeter (Molecular Devices) with an excitation wavelength of 580 nm, an emission wavelength of 630, nm and a bandpass filter at 600 nm. The signal was determined for each well by taking a zero time point immediately after initiating the reaction and at 30 minutes.

Key Research Accomplishments

High throughput assay development and small molecule library screening

- Purification of large quantities of proteins (100-300 mg each) for use in high throughput screening and secondary studies, including Dbs, Tiam1, Sos1, RhoA, RhoB, RhoC, Cdc42, Rac1, and H-Ras
- Screening ~30,000 compounds for activity as chemical modulators of Dbs-mediated activation of RhoA through a collaboration with Dr. Ross Stein, Laboratory for Drug Discovery in Neurodegeneration, Harvard University

Reportable Outcomes

N/A

Conclusions

In conclusion, recent studies have further linked Rho GTPase activation to cancer progression and acquisition of a metastatic phenotype in many types of cancers including breast cancers [7-19]. Therefore, we have initiated a high throughput approach with the aim of identifying small molecule inhibitors that may be characterized using biochemical, as well as cellular methods.

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Appendices
N/A